Author's response to reviews

Title: Lubiprostone ameliorates the cystic fibrosis mouse intestinal phenotype

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Author's response to reviews: see over
Response to Referees' Comments

Referee 1:

Referee 1 had no comments on the revised manuscript.

Referee 2:

1. As Dr. De Jonge stated in his review, the main intestinal phenotype of CFTR -/- mice is intestinal obstruction and decreased survival. The phenotypic changes reported by the authors, such as bacterial overgrowth and delayed intestinal transit although present, are less well characterized.

We agree that intestinal obstruction and poor survival are the major phenotypes in the CF mouse. We have shown that SIBO contributes to excessive mucus accumulation (ref.18) and there is also a relationship between SIBO and impaired intestinal motility (ref.17). Therefore, inclusion of these aspects of the CF phenotype are relevant.

2. Although the literature is divided about the mechanism of action of lubiprostone with regard to intestinal anion secretion, the preponderance of the data strongly favors lubiprostone acting as a local prostaglandin that activates CFTR in most cases. I am aware of few in situ data obtained from intact organisms that support the alternative explanation.

The goal of this work was not to resolve the dispute about the mechanism of lubiprostone's actions. Because the issue is unsettled, we needed to entertain the different possibilities currently being debated in interpreting our data.

3. The authors make several statements stating that lubiprostone "may" increase bicarbonate secretion, even this was clearly shown in rats in ref. 20.

With respect to ref.20, we state in the Discussion: "Lubiprostone has been shown to stimulate duodenal bicarbonate (HCO3-) secretion [20] and HCO3-ion supports efficient intestinal mucin release [31]." and "Lubiprostone has been reported to stimulate bicarbonate secretion which was suggested to be through activation of the EP4 PGE2 receptor [20] and activation of Cl- secretion was blocked by an EP4 receptor antagonist [23]." We did not use the word "may" in this context, and we are not sure what the referee means by this comment.

4. The authors use rhodamine dextran to measure intestinal transit. Although the authors state that it is a "nondigestible, nonabsorbable tracer", dextran is fermented by colonic flora into butyrate (Olano-Martin E, Mountzouris KC, Gibson GR, Rastall RA. In vitro fermentability of dextran, oligodextran and maltodextrin by human gut bacteria. Br J Nutr. 2000 83:247-55). Given the marked differences of intestinal bacterial colonization between the groups, would the use of this marker add artifact to the measurements?

We reported in ref. 28 that recovery of the fluorescent dextran from the gut lumen was +/-10% of the gavaged amount. Also, in the reference cited by the Referee, dextran was a poor substrate for bacteria being only ~10-15% utilized after 6 hrs of fermentation (Fig.4 of the cited work). Therefore, this is unlikely to be an issue in our work.
5. The authors speculate that some of their findings may be due to changes in the hydration status of mucus. Yet, mucus in biological systems is considered to be 99% hydrated. If the authors are aware of intestinal mucus in healthy living animals that is not fully hydrated, these data should be cited.

It is well appreciated that the rheology of mucus in CF is significantly altered and it has been shown that the water content of CF mucus is less than in healthy individuals, as reviewed in Adv Drug Deliv Rev. 2009 February 27; 61(2): 86–100.

6. Since the authors did not measure immune cell function but rather reported expression changes of immune-function related genes, I reiterate my prior comment that the data should not be reported as the "innate immune response" or equivalent. Lacking direct measurements of mast cell migration or actual measures of immune function, it is exceedingly difficult to interpret expression changes of this nature.

We stand by our previous response to this comment.

7. The Discussion is overly long and speculative and would thus benefit from deletion of many of its sections, despite the authors statements to the contrary.

We stand by our previous response to this comment.

Minor Essential Revisions
The authors should consider combining Figs. 2 and 3
Noted.

Referee 3:

1. p. 5 para. 3 (revised text): “However, at high concentrations, lubiprostone can also activate the CFTR Cl- channel”. The same statement based on incorrect quotation or untidily reading of the 2009 Gastroenterology paper by Bijvelds et al (ref. 23) can be found at several places in the recent review by Dr. Woods (ref.19). In reality, the EC50 for lubiprostone activation of CFTR in T84 cells and in non-CF mouse and human ileal epithelium reported in the Gastro paper (e.g. see p. 982) is appr. 50 nM, i.e. not so different from the “less than 100 nM concentrations required to activate ClC-2-like channels” in ref. 39, the EC50 reported by Cuppoletti et al. in T84 cells (appr. 20 nM), and the EC50 of 43.5 and 31.7 nM in guinea pig small intestine and colon (ref. 24). The EC50 of 50 nM reported in ref. 23 is certainly far below a “potency in the micromolar range”, as erroneously stated in ref. 19. Therefore, CFTR activation by lubiprostone in the intestine is not an artefact of suprapharmacological dosages, but is likely to occur in patients undergoing oral treatment with this drug. In conclusion, the term “at high concentrations” is misleading and should be omitted from the text.

In ref.39, there was a pretty large difference in potency of lubiprostone for activating Clc2-like channels vs. CFTR-like channels. In that study, they used the term K1/2, meaning the half-activating dose. They showed that the "... K1/2 for ClC-2 is 69 +/- 18.8 nM whereas for CFTR the K1/2 is 791 +/- 273 nM. These K1/2 values are significantly different from one another (P<0.01)."

So, there is more than a 10-fold difference in the half-activating dose of lubiprostone for Clc2 vs. CFTR in A6 cells. Also, almost all the channels with Clc2 properties were activated at 100 nM lubiprostone whereas only about 20% the CFTR-like channels were activated at this concentration. It may be
that the difference in potency in that study is due to species differences, because the A6 cells used are a Xenopus kidney cell line. In any case, we have removed the qualifier ‘at high concentrations’ from the manuscript.

2. p. 5 para 3 (revised text): ref. 23 is also quoted for the suggestion that “lubiprostone-induced transport is completely CFTR-dependent”. This is only true for the Cl-secretory component of intestinal ion transport in the crypts, but certainly not for the NaCl and fluid absorptive component of transport in the intestinal villi. In fact, on p. 984 of ref. 23 it is suggested that lubiprostone, through its ability to trigger EP4-cAMP-PKA signalling, might inhibit electroneutral absorption of NaCl at the level of NHE3 and could therefore act anti-absorptive rather than pro-secretory in CF patients. Such a model might also help to explain the beneficial effects of lubiprostone reported in a recent case study (ref. 18). Therefore, the quotation that lubiprostone-induced transport is completely CFTR-dependent should be modified.

We agree this was poorly worded and incorrect. This statement has been modified to read "... lubiprostone-induced chloride secretion is completely CFTR-dependent [23]".

3. Author’s responses to referee 3, comment 1: It is still highly regrettable that the authors did not pursue their original plans to study possible beneficial effects of in vivo lubiprostone treatment on survival of Cftr-/- mice from intestinal obstruction “for ethical reasons”. Even negative results would be very important for the field, considering the controversies around lubiprostone action. But there is a fair chance that lubiprostone, either though CIC-2 activation (if Cuppoletti et al are correct) or though inhibition of salt and water absorption (ref. 23; see above) might increase the fluidity of the intestinal lumen and lower mortality by intestinal obstruction. The effect of lubiprostone treatment might be similar to the effect of crossing Cftr-/- mice with NHE3+/- mice (having lost ~50% of their salt absorptive capacity), resulting in a dramatic improvement of the CF phenotype (see Bradford EM et al 2009 Am J Physiol 296: G886-G898).

Noted.